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(54) Title: CARIES INHIBITION

(57) Abstract

Compositions for the inhibition of caries and gingivitis, containing a protein or polypeptide particularly phosphoproteins or polyphosphopeptides including those containing the amino acid sequence $(X\text{-}Y\text{-}Z)_n$ where X and Z are a phosphoserine, phosphothreonine, phosphotyrosine, glutamate or aspartate, Y is any amino acid and n is 1 or more. Particular examples of suitable active ingredients are sodium caseinate, calcium caseinate and phosvitin. Suitable proteins or polypeptides were tested via the dissolution rate of hydroxyapatite as measured by the rate of calcium and phosphate released from a hydroxyapatite column.

'CARIES INHIBITION'

This invention relates to caries inhibiton. The invention also relates to gingivitis inhibition.

The present invention provides an orally ingestible composition containing a caries and gingivitis inhibiting amount of a protein or a polypeptide or a salt thereof.

Preferably, the protein or polypeptide is a phosphoprotein or a polyphosphopeptide.

Preferably, the protein or polypeptide is an acidic phosphoprotein or polypeptide.

Preferably, the protein or polypeptide contains the amino acid sequence (X-Y-Z) where X and Z are a phosphoserine, phosphothreonine, phosphotyrosine, glutamate or asparate and Y is any amino acid.

Preferably, the protein or polypeptide contains a plurality of units each having the amino acid sequence (X-Y-Z) where X, Y and Z are as stated in above.

Preferably, the protein or polypeptide contains a group of formula (X-Y-Z)n where X, Y and Z are as stated in claim 4 and n is 1 or more.

Preferably, n is 3 or more.

Preferably, X and Z are phosphoserine.

Preferably, the protein or polypeptide is a polyphosphoserine.

Preferably, the phosphate groups of the polyphosphoserine are spaced at about 6.88 Angstrom Units.

Preferably, the protein is a casein.

Preferably, the protein is alpha-s-casein.

Alternatively, the protein is phosvitin.

Preferably, the protein or polypeptide is in solution.

Preferably, the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 45 nmol/min under the test conditions defined herein.

Preferably, the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 80 nmol/min under the test conditions defined herein.

Preferably, the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 90 nmol/min under the test conditions defined herein.

Preferably, the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 95 nmol/min under the test conditions defined herein.

Preferably, the protein or polypeptide is present as up to 10% by weight.

Preferably, the protein or polypeptide is present as up to 5% by weight.

Preferably, the protein or polypeptide is present as up to 2% by weight.

Preferably, the composition additionally contains urea.

The composition of this invention may be in the form of a foodstuff, confectionery, dentifrice, tablet or comprise a pharmacologically acceptable vehicle, solution of suspension for topical application to the teeth or a mouthwash. Other modes of administering the protein or polypeptide would be acceptable if pharmacologically acceptable.

Of particular interest as compositions are chewing gum, breakfast foods, ice-cream and other frozen confectionery, confectionery, sweets and cakes as these are all known as caries problem foods.

Also of particular interest are dentifrices, mouthwashes and preparations for topical application to teeth.

α_s- casein and other caseins are obtainable from milk, phosvitin is obtainable from egg yolks and other suitable phosphoproteins include those which are obtainable from

cereals, nuts and vegetables particularly in bran husks or sheaths. In particular, rice, wheat, oat, barley and rye brans are a source of such phosphoproteins.

Polypeptides of interest include polyphosphoserine, polyglutamate and polyaspartate.

The present invention also provides a process of inhibiting dental caries and/or tooth erosion and/or gingivitis comprising applying to the teeth with a carrier a cariostatic and/or antigingivitis agent selected from a protein or a polypeptide or a salt thereof.

The present invention also provides a first test procedure for selecting among the proteins or polypeptides that may be used those that are most effective.

TEST 1

The purpose of this test is to determine hydroxyapatite dissolution and in this respect since tooth enamel is largely composed of hydroxyapatite it is believed that useful comparisons can be made.

Double distilled, deionised water (greater than 10 MΩ/cm) was used throughout. Analytical reagent grade chemicals were obtained from Ajax Chemicals, Australia. Hydroxyapatite-spheroidal was purchased from BDH, and phosvitin from Sigma Chemical Co., Missouri, U.S.A. The milk proteins were fractionated by the method of Zittle and Custer (1), and their purity assessed by polyacrylamide gel electrophoresis using a modification of the method of Groves and Kiddy (2).

Methods

Hydroxyapatite Dissolution Rate Assay

A chromatography column (pharmacia K9/15) containing 1 g. of hydroxyapatite beads was used for the demineralisation assay. Tris 5 mM, pH 8.3 containing 50 mM NaCl and 20 mg/l neomycin was used as the column influent buffer at 20°C and at a rate of 1.000±0.003 ml./min. A protein solution (10 mg. 10 ml. of influent buffer) was applied to the column and 1 ml. fractions were collected before and after protein application

and assayed for total calcium, phosphate and protein. From these values a rate of dissolution (nmol calcium or phosphate per min) for each 1 ml. fraction was obtained.

Calcium, Phosphate and Protein Assays

Inorganic phosphate was measured by the method of Itaya and Ui(3) and protein by the method of bradford (4). The determination of calcium was by atomic absorption spectrophotometry using 1% lanthanum chloride to prevent phosphate suppression.

Results

The proteins used for the study are listed in Table 1. they are all acidic proteins and included four phosphoproteins and three non-phosphorylated proteins from the whey fraction of bovine milk. The effect of the individual proteins on hydroxyapatite dissolution rate is shown in Table 2.

Table 1. Properties of various phosphorylated and non-phosphorylated acidic proteins.

Protein	Molecular Weight	Phosphoserine Content [a]	Isoelectric Point	Carbohydrate Content
Phosvitin	35,500	110	1.5	+
α_s-casein	23,613	8	4.1	-
β-casein	24,020	5	4.5	-
κ-casein	19,037	1	3.7	+
α-lactalbumin	14,174	-	5.1	-
β-lactaglobulin	18,362	-	5.3	-
Bovine serum albumin	66,210	-	4.7	-

a. Residues per mol protein

Table 2. Effect of phosphorylated and non-phosphorylated proteins on hydroxyapatite dissolution rate.

Protein	Reduction in calcium dissolution rate[a] (nmol/min)	Reduction in phosphate dissolution rate (nmol/min)	Amount of protein bound (mg)
Phosvitin	93.1± 5.4[b]	63.8± 9.4	1.87±0.62
α_s-casein	100.1± 4.1[b]	63.5± 3.3	5.58±0.03
β-casein	94.8±11.7[b]	64.0±19.3	7.45±0.37
κ-casein	56.3± 8.9	33.7± 6.8	4.17±0.26
α-lactalbumin	2.7± 1.7	2.9± 0.6	0.48±0.17
β-lactoglobulin	17.1± 1.7	12.5± 1.2	1.80±0.71
Bovine serum albumin	31.6± 4.5	20.5± 3.3	2.09±0.05

a. means±SD, n = 3

b. not significantly different P>0.5

In a trial of the above test the dissolution rate of hydroxyapatite as measured by the rate of calcium and phosphate released from the hydroxyapatite column was constant over a two hour period calcium 353.6±3.9 nmol/min, phosphate 225.4±6.8 nmol/min. The dissolution rates obtained using different hydroxapatite columns showed greater variation, calcium 354.2±23.8 nmol/min, phosphate 229.6±30.8 nmol/min, n = 11. This intercolumn variation in dissolution rate could be attributable to different column packing resulting in a different HA surface area exposed.

The effect of phosvitin on the dissolution rate of hydroxyapatite is shown in Figure 1. The protein caused an initial increase in the dissolution rate of phosphate which then decreased and approached a new steady state level; 63.8 nmol/min less than the rate prior to phosvitin application. The protein caused an immediate and marked drop in the calcium dissolution rate which then increased and approached a steady-state level 93.1 nmol/min less than that prior to phosvitin application. The amount of protein that passed through the

column was measured and from this the amount retained was calculated 1.87 mg. The dissolution rate returned to the original value after phosvitin was eluted from the column with 10 ml of eluent buffer containing 1.5 M phosphate, followed by buffer not containing phosphate.

The trace for α_s-casein was very similar to that of phosvitin except that the immediate drop in calcium dissolution rate was not as marked Fig. 2. The difference in the steady-state rates of calcium and phosphate released before and after α_s-casein application were very similar to those in Figure 1 (calcium, 100.1 nmol/min. phosphate 63.5 nmol/min).

The results obtained for β-casein (Fig. 3) were characteristic of all the other proteins tested, except for the final steady-state rates of calcium and phosphate released. All proteins (with the exception of phosvitin and α_s-casein) caused an initial increase in the dissolution rate of both calcium and phosphate which then decreased as the proteins passed out of the column. The mean differences between the steady-state dissolution rates before and after protein application, together with the amount of protein bound, for all proteins tested is presented in Table 2 above. The results show that the four phosphoproteins gave a marked reduction in the steady-state dissolution rate of HA with phosvitin, α_s-casein and β-casein all giving the same reduction in calcium and phosphate dossolution.

The results show that all the acidic proteins caused a reduction in the steady-state dissolution rate of hydroxyapatite. However, the greatest reduction was given by the four phosphoproteins; phosvitin, α_s-casein, β-casein and to a lesser extent κ-casein.

From the above and from other data which suggests that adjacent phosphate groups of polyphosphoserine compounds have a spacing of about 6.88 Angstrom units when in a beta-pleated sheet configuration and that calcium atoms in a hydroxyapatite surface along the c-axis will also be spaced at about 6.88 Angstrom units, we speculate that a phosphate group-calcium atom bond materially reduces hydroxyapatite dissolution.

References:

1. Zittle, C.A., Custer, J.H.: Purification and some properties of α_s-casein, J. Dairy Sci 46: 1183-1189, 1963.

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3. Itaya, K., Ui, M.: A new micromethod for the colorimetric determination of inorganic phosphate. Clin, Chim, Acta 14: 361-366, 1966.

4. Bradford, MM.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254, 1976.

TEST 2

For the purpose of in vivo testing, the following test procedure for determining the effect of casein and whey protein on caries incidence in the Sprague-Dawley rat.

Materials and Methods.

Forty-five, weanling, male Sprague-Dawley rats, 18 days old, bred from animals fed a fluoride free diet were used. The rats were marked for indentification and then randomly distributed with respect to diet. They were housed in raised-bottom stainless-steel cages, one group of 15 per cage and fed a powdered cariogenic diet with either deionised water (control), a 2% casein solution or a 2% whey protein solution _ad libitum_. The cariogenic diet was a modified MIT - 2ØØ diet shown in Table 3.

Table 3 - Composition of modified MIT - 200 cariogenic diet

Component	% wt
Sucrose [a]	67
Wheyprotein Concentrate	20
Salt mixture [b]	3
Cottonseed oil	3
Cellulose [a]	6
Vitamin mixture [a, b]	1

a. Calcium and phosphate not detectable, fluoride content of complete diet was less than 0.2 g per g dry weight.
b. Vitamin and Salt mixture described in detail elsewhere.

The animals were weighed daily and the amounts of powdered diet and liquid consumed over a 24 h period by each group was measured. After 42 days on the diet, the animals were killed by cervical dislocation and treated as described below.

Caries evaluation

Fissure caries was assessed using the method of Konig, Marthaler and Muhlemann (1958)(5). The mandible was removed from each rat and placed in formol-saline. The jaws were the sectioned and stained by the method of Konig et al (1958)(5), as described by Green and Hartles (1966)(6) to provide series of 100 μm thick longitudinal mesio-distal sections of the molar teeth. Only the main fissures of the first and second molar teeth were assessed for caries.

Results

Diet Consumption

The relative consumption of solid and liquid diet by the three groups of rats was tested by an analysis of variance (by diet). This showed that the quanitites of both solid and liquid consumed by each group were not significantly different ($P>0.75$).

Caries Analysis.

The caries data shown in Table 4 were analysed in an analysis of variance table by diet.

Table 4

Caries Experience Data

Animals	Number of carious fissures [a]
Control Group	7.92±2.06
Casein Group	1.87±2.50
Whey Protein Group	4.73±3.85

a. Maximum number possible 10.

The animals drinking the 2% casein solution had 76.5% less carious fissures than the control animals ($p<0.001$), and the animals consuming the 2% whey protein solution had 40.3% less carious fissures than the control group ($p<0.01$). The correlation of caries incidence with the final weight of the rat was tested for the three groups. No correlation attained significance ($p>0.1$).

Similarly, the initial and final weights showed no correlation, nor were weight gain and caries incidence correlated.

Conclusion

Acidic proteins in the drinking water substantially reduced caries incidence of male Sprague-Dawley rats, however the phosphorylated protein (casein) caused a greater reduction than the non-phosphorylated whey proteins.

References:

(5) Konig K.G., Marthaler T.M. and Muhlemann H.R. 1958: Methodik der Kurzfristig erzeugten Rattenkaries. Dr. Zahn-Mund-u. Kieferheilk. 29, 99-127.

(6) Green R.M. and Hartles R.L. 1966: The effect of differing high carbohydrates diets on dental caries in the albino rat. Br. J. Nutr. 20, 317-323.

TEST 3

This test was to determine the effect of protein on the adsorption of the bacterium Streptococcus mutans to hydroxyapatite.

Materials and Methods

Hydroxyapatite discs were prepared by pressing 15Ø mg of hydroxyapatite (Bio-Gel HTP, Biorad Laboratories) for 5 min under 5 tons of pressure in a KBr press. The discs were hydrated then incubated with either various protein solutions or imidazole buffer (Ø.Ø5M pH 7.Ø, containing Ø.Ø25 M NaCl). The adherence of ^{3}H-labelled S.mutans PK1 cells was studied by incubating the pretreated discs with ^{3}H-thymidine labelled cells ($1Ø^9$ cells/ml) suspended in the imidazole buffer. The protein solutions used were all 5 mg/ml in imidazole buffer. The proteins and polypeptides studied were α_s-casein, β-casein, κ-casein, phosvi n, bovine serum albumin, histone III, histone VIII, α-lactalbumin, B-lactoglobulin, poly-l-lysine and poly-l-glutamate. The caseins were prepared by selective precipitation and the other proteins were purchased from Sigma Chemical Co., Missouri, U.S.A.

Results

The effect of pretreating hydroxyapatite discs with various protein solutions on the adherence of S.mutans cells is shown in Table 5.

Table 5. Effect of protein on S. mutans adherence to hydroxyapatite.

Proteins	Type	Number of S.mutans cells adsorbed (x10^7)
Control	-	1.9±Ø.6[a]
α_{s1}-casein	acidic phosphoprotein	Ø.5±Ø.3
β- casein	acidic phosphoprotein	Ø.4±Ø.4
κ- casein	acidic phosphoprotein	Ø.6±Ø.1
phosvitin	acidic phosphoprotein	Ø.5±Ø.1
BSA	acidic protein	Ø.6±Ø.1
histone III	basic protein	2.2±Ø.9
histone VIII	basic protein	2.6±0.9

Proteins	Type	Number of S.mutans cells adsorbed ($\times 10^7$)
-lactalbumin	acidic protein	1.0±0.4
-lactoglobulin	acidic protein	0.9±0.4
poly-lysine	basic polypeptide	3.8±1.1
poly-glutamate	acidic polypeptide	0.5±0.1

a means±SD,n=8

Conclusion

All the acidic proteins and polypeptides (especially the phosphoproteins) caused a reduction in bacterial adherence to hydroxyapatite. However, the basic proteins and polypeptides either had no effect or enhanced bacterial adherence to hydroxyapatite.

Having regard to the successful results obtained from using the above tests Applicants have formulated various compositions in accordance with this invention as follows. In general, the compositions contain from 0.5-20% by weight of protein or polypeptide.

Example 1. Flour: In a device for mixing dry substances, 1% by weight of powdered sodium caseinate was blended with flour.

Example 2. Cereal: A breakfast cereal was sprayed with a solution of calcium caseinate in water. The cereal flakes were then dried to produce a finished product containing 1% calcium caseinate.

Example 3. Bread: 2% by weight of calcium caseinate was added to the flour during the mixing of ingredients for the manufacture of bread.

Example 4. Cake mix: 1% by weight of calcium caseinate was added to the dry ingredients in the preparation of a cake mix.

Example 5. Confectionery: In the preparation of confectionery 2% by weight of calcium caseinate was added to the final mixture.

Example 5. Biscuit: In the preparation of a biscuit mixture 5% by weight of calcium caseinate was added to the other dry ingredients during mixing.

Example 7. Beverage: A beverage was prepared in which 1% weight of calcium caseinate had been dissolved.

Example 8. Tablet: A tablet was made containing 10% by weight of calcium caseinate together with excipients being flavouring matter and binding material.

In preparation of a typical dentifrice within the scope of this invention, the requisite salt and salts of the selected protein or polypeptide are incorporated into dentifrice compositions in any suitable manner depending on whether a powder, paste or liquid preparation is to be produced. For this purpose appropriate preparations of surface-active agents, binders, flavouring materials and other excipients required to achieve the required form of dentifrice are added.

The invention is further illustrated by the following examples: Example 9. <u>Tooth paste</u>: A toothpaste was prepared having the following composition:-

Calcium caseinate	5.0% by weight
Gum tragacanth	1.0% " "
Saccharin	0.1% " "
Glycerin (B.P.)	20.0% " "
Sodium lauryl sulphate	1.0% " "
Methyl parahydroxy benzoate	0.1% " "
Flavouring and colouring	1.0% " "
Dibasic calcium phosphate	35.0% " "
Water	36.8% "

Example 10. Toothpaste: A preparation as set out in example 9 was repeated but with the addition of 2% sodium fluoride in a suitable form.

Example 11. Toothpaste: A preparation as set out in example 9 was repeated but with the addition of 0.4% stannous fluoride in a suitable form

Example 12. Toothpaste: A preparation as set out in example 9 was repeated but with the addition of 0.1% mono sodium fluorophosphate in a suitable form.

Example 13. Tooth powder: The following preparation was made:-

Calcium caseinate	5.0% by weight
Soluble saccharin	0.1% " "
Colour agent	Trace " "
Dibasic calcium phosphate	94.1% " "

Example 14. Tooth powder: A preparation as set out in example 13 was made but with the addition of 1% mono sodium fluorophosphate in a suitable form.

Example 15. Liquid dentifrice: A preparation was made consisting of:-

Sodium alginate	1.5% by weight
Calcium caseinate	5.0% " "
Sodium lauryl sulphate	1.0% " "
Flavouring	Trace " "
Colouring	Trace " "
Water	92.5% " "

pH adjusted to 7.0

Example 16. Liquid dentifrice: As for example 15 but with 0.5% sodium fluoride added.

Example 17. Mouthwash: The following preparation was made:-

Sodium caseinate	2.0% by weight
Sodium fluoride	0.5% " "
Flavouring	Trace " "
Colouring	Trace " "
Water	97.5% " "

In the above, casein was used princiaplly because of economics but in lieu phosvitin or other material might be used.

The claims form part of the disclosure of this specification.

Modifications and adaptations may be made to the above described without departing from the spirit and scope of this invention which includes every novel feature and combination of features disclosed herein.

1. An orally ingestible composition containing a caries and gingivitis inhibiting amount of a protein or a polypeptide or a salt thereof.

2. A composition as claimed in claim 1, wherein the protein or polypeptide is a phosphoprotein or a polyphosphopeptide.

3. A composition as claimed in claim 1, wherein the protein or polypeptide is an acidic phosphoprotein or polypeptide.

4. A composition as claimed in claim 1, wherein the protein or polypeptide contains the amino acid sequence (X-Y-Z) where X and Z are an phosphoserine, phosphothreonine, phosphotyrosine, glutamate or asparate and Y is any amino acid.

5. A composition as claimed in claim 4, wherein the protein or polypeptide contains a plurality of units each having the amino acid sequence (X-Y-Z) where X, Y and Z are as stated in claim 4.

6. A composition as claimed in claim 5, wherein the protein or polypeptide contains a group of formula $(X\text{-}Y\text{-}Z)_n$ where X, Y and Z are as stated in claim 4 and n is 1 or more.

7. A composition as claimed in claim 6, wherein n is 3 or more.

8. A composition as claimed in any one of claims 4-7, wherein X and Z are phosphoserine.

9. A composition as claimed in any one of claims 4-8, wherein the protein or polypeptide is a polyphosphoserine.

10. A composition as in claim 9, wherein the phosphate groups of the polyphosphoserine are spaced at about 6.88 Angstrom Units spacing.

11. A composition as claimed in any preceding claim, wherein the protein is a casein.

12. A composition as claimed in claim 11, wherein the protein is alpha-s-casein.

13. A composition as claimed in any one of claims 1-10, wherein the protein is phosvitin.

14. A composition as claimed in any preceding claim, wherein the protein or polypeptide is in solution.

15. A composition as claimed in any preceding claim, wherein the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 45 nmol/min under the test conditions defined herein.

16. A composition as claimed in any one of claims 1-14, wherein the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 80 nmol/min under the test conditions defined herein.

17. A composition as claimed in any one of claims 1-14, wherein the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 90 nmol/min under the test conditions defined herein.

18. A composition as claimed in any one of claims 1-14, wherein the protein or polypeptide is one exhibiting a reduction in calcium dissolution rate of at least 95 nmol/min under the test conditions defined herein.

19. A composition as claimed in any preceding claim, wherein the protein or polypeptide is present as up to 10% by weight.

20. A composition as claimed in any preceding claim, wherein the protein or polypeptide is present as up to 5% by weight.

21. A composition as claimed in any preceding claim, wherein the protein or polypeptide is present as up to 2% by weight.

22. A composition as claimed in any preceding claim, and additionally containing urea.

23. A composition as claimed in any preceding claim, in the form of a dentifrice mouthwash, tablet, lozenge or capsule.

24. A composition as claimed in any one of claims 1-22 in the form of a foodstuff.

25. A composition as claimed in any one of claims 1-22 in the form of confectionery.

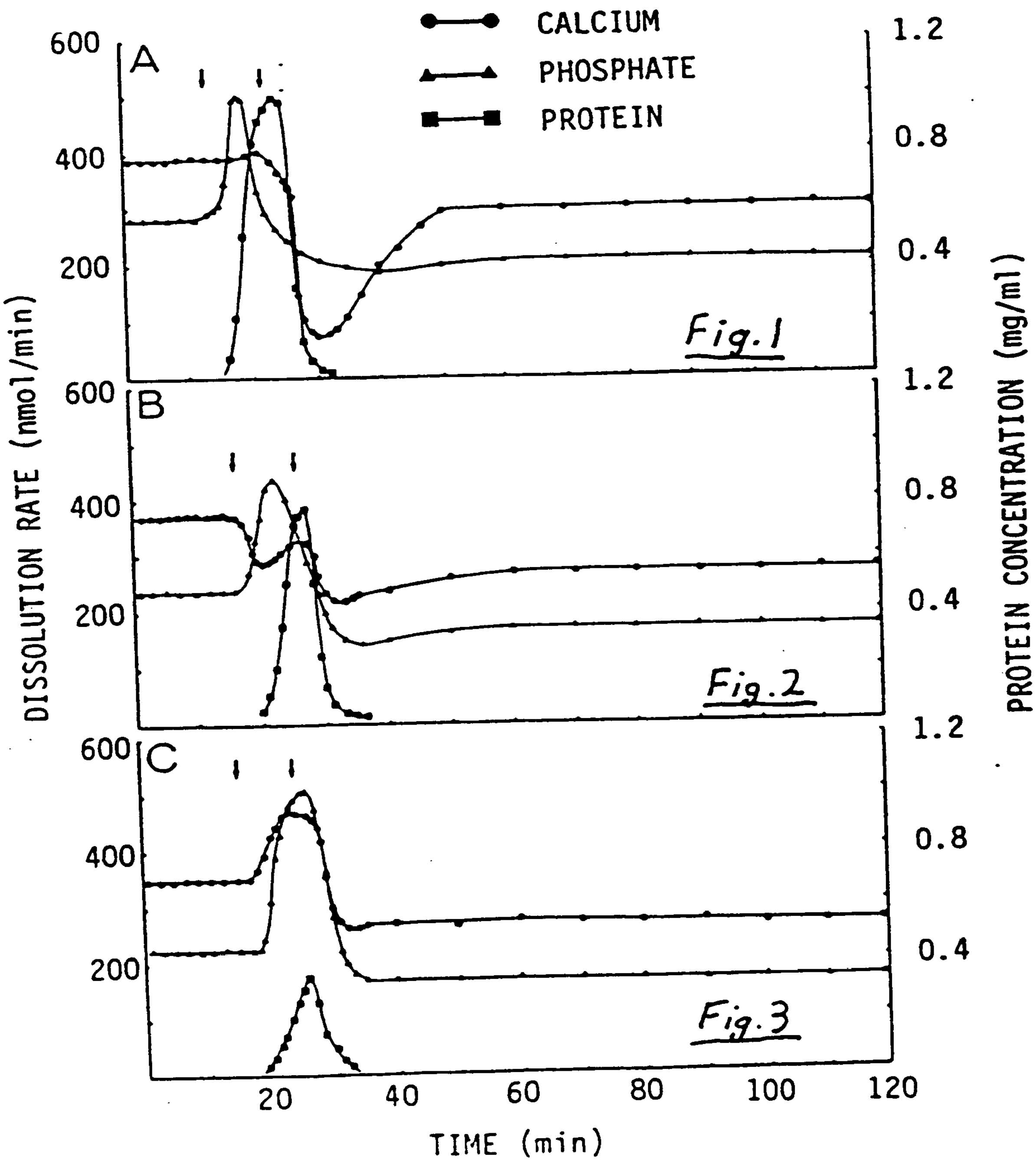
26. An orally ingestible composition substantially as hereinbefore described with reference to any one of the Examples.

27. A process of inhibiting dental caries and/or tooth erosion and/or gingivitis comprising applying to the teeth with a carrier a cariostatic and/or anti-gingivitis agent selected from a protein or a polypeptide or a salt thereof.

28. A process of inhibiting dental caries and/or tooth erosion and/or gingivitis comprising applying to the teeth a composition in accordance with any one of claims 1-26.

29. A composition as claimed in any preceding claim wherein the protein or polypeptide is present as 1% by weight or greater.

30. The articles, things, parts, elements steps, features, methods, processes, compounds and compositions referred to or indicated in the specificaiton and/or claims of the application individually or collectively, and any and all combinations of any two or more of such.


CALCIUM
PHOSPHATE
PROTEIN
A
B
C
Fig. 1
Fig. 2
Fig. 3
600
400
200
1.2
0.8
0.4
20
40
60
80
100
120
DISSOLUTION RATE (nmol/min)
PROTEIN CONCENTRATION (mg/ml)
TIME (min)